

## Activity of T-705 in a Hamster Model of Yellow Fever Virus Infection in Comparison with That of a Chemically Related Compound, T-1106<sup>∇</sup>

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Received 8 August 2008/Returned for modification 26 September 2008/Accepted 15 October 2008

Treatment with the nucleoside analog T-1106 was previously shown to be effective in a hamster model of yellow fever virus (YFV) disease, even though it had only slight activity in cell culture. In the study described in this report, the activity of T-705, a chemically related compound currently undergoing clinical trials for the treatment of influenza (FDA News 4:1, 2007), was tested against YFV in cell culture and in the hamster model. The antiviral efficacy of T-705 in cell culture occurred at a concentration of 330  $\mu$ M, which was more than threefold lower than the concentration at which T-1106 had antiviral efficacy, as determined by a virus yield reduction assay and confirmed by a luciferase-based ATP detection assay. Time-of-addition studies revealed that addition of T-705, T-1106, or ribavirin at 0, 4, 8, or 12 h after virus challenge was effective in inhibiting virus in Vero cells, suggesting that these three agents have similar mechanisms of action in cell culture. Because of its more potent activity in cell culture, it was anticipated that T-705 treatment of hamsters infected with YFV would result in protection from disease. Significant improvements in survival and disease parameters were seen in infected animals when T-705 was administered orally at a dose of 200 or 400 mg/kg of body weight per day when it was given twice a day for 8 days. Significant improvements were also observed with a dose of 400 mg/kg/day when treatment initiation was delayed as late as 3 days after virus inoculation. Although the dose of T-705 required for efficacy in hamsters is higher than that of T-1106 required for efficacy, T-705 treatment is effective in significantly improving disease parameters in YFV-infected hamsters, which may indicate its potential utility in the treatment of YFV disease in humans.

Yellow fever virus (YFV) is a flavivirus that is spread by mosquitoes in areas of endemicity in Africa and South America. Despite the availability of an effective vaccine, this virus continues to cause periodic outbreaks and significant morbidity and mortality (16, 24). Cases of clinical disease after adverse vaccination events have also been reported, resulting in life-threatening illness (3, 9, 12, 13, 25). Cases of YFV disease outside the natural range have been documented and were contracted after travel into the natural range of YFV (1, 14, 24). Although there is a great need for therapies, no antiviral agents are approved for use for the treatment of YFV disease.

The rhesus macaque (*Macacus rhesus*) has been used as a model of YFV infection and disease. Infection of these primates usually results in a fulminating disease similar to fatal cases of the disease in humans (2, 15, 17). Due to the increased restrictions on the use of primates as laboratory models, a small-animal model of viscerotropic YFV disease was needed. A hamster model of YFV disease has recently been characterized, and the infection in hamsters is similar in many regards to that in humans (20, 23, 26). This hamster model has served as a useful tool for the evaluation of antiviral agents for the treatment of YFV disease (11, 19). The hamster-adapted Jimeenez strain of YFV used in this model causes disease and death after intraperitoneal (i.p.) injection of hamsters (23). Useful parameters for the evaluation of antiviral compounds include

the rate of survival; the YFV titer in the liver; serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels; weight change; and several serum parameters, including elevated bilirubin and blood urea nitrogen levels (10, 11, 19).

T-1106, a pyrazine nucleoside analog, was recently shown to significantly improve the rates of survival and disease parameters in hamsters infected with YFV (10). Doses as low as 32 mg/kg of body weight per day (120  $\mu$ mol/kg/day) were effective against the virus. Despite the lack of cell culture activity at concentrations below 100  $\mu$ g/ml (369  $\mu$ M), testing was performed with the hamster model because of activity against the polymerase of hepatitis C virus in vitro (7). T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide), a substituted pyrazine compound that is chemically similar to T-1106 (Fig. 1), has been shown to have potent activity in vitro and in vivo against influenza virus and other RNA viruses (5, 6, 8, 21, 22). This compound is also currently undergoing phase II clinical trials for the treatment of human influenza virus infection. The purpose of this study was to compare the efficacy of T-705 with that of T-1106 in cell culture and in the hamster model.

### MATERIALS AND METHODS

**Animals.** Female Syrian golden hamsters (average weight, 100 g) were used. After a 24-h quarantine period and a 7-day acclimation period, the animals were randomly assigned to cages and were individually marked with ear tags. All work with these animals was performed in the biosafety level 3 area of the AAALAC-accredited Laboratory Animal Research Center at Utah State University.

**Test articles.** T-705 and T-1106 were provided by Toyama Chemical Company, Ltd. (Tokyo, Japan). T-705 was suspended in 0.4% carboxymethyl cellulose at various concentrations for oral (p.o.) treatment. T-1106 was dissolved in saline or 0.4% carboxymethyl cellulose and was administered by intraperitoneal (i.p.)

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<sup>∇</sup> Published ahead of print on 27 October 2008.

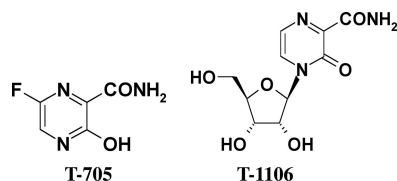


FIG. 1. Structures of T-705 and T-1106.

injection and p.o. treatment, respectively. Ribavirin was provided by Valeant Pharmaceuticals (Costa Mesa, CA) and in these studies was used as a positive control at a dose of 50 mg/kg/day prepared in sterile saline. Interferon alfacon-1 (infergen), a consensus-type interferon, was provided by Lawrence Blatt (InterMune, Inc., Brisbane, CA) as an aqueous solution and was used as a positive control in the cell culture assays.

**Virus.** The Jimenez strain, a hamster-adapted strain of YFV, was obtained as a generous gift from Robert B. Tesh (University of Texas Medical Branch, Galveston). The virus was inoculated i.p. into five adult female hamsters. The livers of the infected hamsters were removed 3 days after virus injection (days postinjection [dpi]) and homogenized in a 2× volume of sterile phosphate-buffered saline. This liver homogenate had a titer of  $10^{6.0}$  50% cell culture infectious doses (CCID<sub>50</sub>s)/ml when it was titrated on Vero cells. The 17D YFV vaccine strain was obtained from the American Type Culture Collection (Manassas, VA), and virus stock was prepared in Vero cells.

**Evaluation of T-705 and T-1106 in cell culture.** The antiviral activities of T-705 and T-1106 were evaluated in Vero cells by cytopathic effect (CPE) inhibition assays and were determined by visual (microscopic) examination of the cells, determination of an increase in the level of neutral red (NR) dye uptake into cells, and virus yield reduction. The effects of eight concentrations of each compound against the 17D strain of YFV were evaluated in 96-well flat-bottomed microplates plated with Vero cells. Vero cells were obtained from the American Type Culture Collection. The compounds were added 5 to 10 min prior to the addition of virus. Virus was added at 2 PFU per well. The test results were read after incubation at 37°C for 6 days. For NR uptake, the NR dye (0.034% in medium) was added to plates after visual examination for 2 h, after which the dye was eluted from the cells and the absorbed dye was quantified. Antiviral activity was expressed as the 50% effective concentration (EC<sub>50</sub>; the amount of drug required to protect 50% of the cells from a virus CPE) or the EC<sub>90</sub>, and a selective index (SI) value was obtained by dividing the 50% cytotoxic concentration (CC<sub>50</sub>) obtained for uninfected cells treated with T-1106 by the EC<sub>90</sub> (21).

The Cell-Titer Glo system (Promega, Madison, WI) was used to determine cell viability by assaying infected and uninfected cells treated with T-705 or T-1106 for the presence of ATP. Appropriate negative and positive controls were used for comparison of the results. Vero cells were plated in a half-growth area of 96-well plates. The assay was performed according to the manufacturer's instructions, and the luminescence was read on an LB960 Cetro luminometer (Berthold Technologies, Oak Ridge, TN).

Time-of-addition studies were also conducted. In those studies, T-705 and T-1106 were added at 0, 4, 8, 12, or 16 h after virus challenge of Vero cells. The compounds were added at concentrations of 1,000, 320, 100, and 32 µg/ml.

Supernatants from each well were collected at 24 h after virus challenge and were analyzed for infectious virus, which corresponded to the virus released in the first round.

**Experimental design for animal studies.** Hamsters were randomly assigned to groups, and 10 to 15 animals were included in each group. Toxicity controls, which consisted of three animals per group, were included to determine if there was any apparent toxicity associated with treatment. Healthy control animals were also included. YFV Jimenez was prepared at a concentration of  $10^{2.0}$  CCID<sub>50</sub>s in minimal essential medium. The hamsters were injected i.p. with 0.1 ml of the diluted virus. Solutions of T-705 and T-1106 were prepared less than 24 h prior to the initial treatment and were kept at 4°C for the duration of the experiment. Animals were treated twice daily for 8 days, with the treatments given 12 h apart. Mortality was observed daily; and the weights were recorded on 0, 3, and 6 dpi. Weight change was determined by the amount of weight lost or gained between 3 and 6 dpi. Serum was taken at 6 dpi to determine the serum ALT and AST levels. To determine the liver virus titer, an additional experiment was conducted in which liver samples were taken at 5 dpi from eight hamsters (from groups of 16 to 23 animals each), and the remaining 8 to 15 animals were left for the evaluation of mortality, weight change, and serum ALT and AST levels. Ribavirin, which was prepared in saline at a dose of 50 mg/kg/day, was used as a positive control compound, and saline was used as a placebo control.

In the first experiment, a simple efficacy study was conducted to determine the effective dose of T-705 in hamsters infected with YFV. Follow-up studies were conducted to determine the minimal effective dose (T-705 was tested at 400, 200, and 100 mg/kg/day) and how long after virus challenge that the drug could be effectively administered (1 through 5 dpi). Various doses of T-1106 were included in these studies for comparative purposes.

**QRT-PCR.** For the detection of YFV RNA levels in the liver, a quantitative real-time PCR (QRT-PCR) was performed as follows. A primer pair (forward primer, AGTTGATTCCATCTTGGGCTTC; reverse primer, ACCTCTTCCTC TCCATCCCATC) and a TaqMan probe (5'-6-carboxyfluorescein-CCTATG GTGGCTCATGGAAGTTGGAAGG-6-carboxy-*N,N,N',N'*-tetramethylrho damine-3') specific for nucleotides 4767 to 4860 of the Asibi YFV vaccine strain (GenBank accession no. AY640589.1) were used (Qiagen, Valencia, CA). The Brilliant QRT-PCR master mix one-step kit (Stratagene, La Jolla, CA) was used for the reverse transcription and amplification of YFV RNA with the primers and probe at 0.2 µM each. One microliter of total cellular RNA (from a total of 100 µl) extracted from infected or control tissues was used. Samples were run on a DNA Engine Opticon 2 apparatus (MJ Research Inc., Waltham, MA). Reverse transcription of the cellular RNA was performed for 30 min at 50°C, followed by PCR, which consisted of 40 cycles of 15 s at 95°C and 60 s at 61°C. The results are given as the relative equivalents, which reflect the amount of YFV RNA present in the sample as extrapolated from a standard curve obtained from amplification of a dilution of total RNA obtained 2 dpi from Vero cells infected with YFV Jimenez. This protocol was previously validated by comparison of the results with those obtained by the infectious cell culture assay (10).

**Assays for serum ALT and AST levels.** Serum was collected antemortem from the ocular sinus of all of the animals in each group. ALT (serum glutamic pyruvic transaminase) or AST reagent (Teco Diagnostics, Anaheim, CA) was used, and the protocol was altered for use in 96-well flat-bottomed microplates, as described previously (10). The plate was then read, and the aminotransferase concentrations were determined according to the manufacturer's instructions.

TABLE 1. Effects of T-705, T-1106, ribavirin, and infergen on YFV 17D infection of Vero cells<sup>a</sup> assayed by visual inspection, NR uptake assay, cell viability (luciferase-based) assay, or virus yield reduction assay

Compound	Unit for concn	EC <sub>50</sub>			CC <sub>50</sub>		EC <sub>90</sub> (VYR <sup>b</sup> )	SI
		Visual inspection of cells	NR dye uptake assay	Luciferase-based cell viability assay	Luciferase-based cell viability assay	NR dye uptake assay		
T-705	µM	180 ± 96	270 ± 0	270 ± 45	>6,370	>6,400	330 ± 90	>19
T-1106	µM	1,080 ± 590	1,800 ± 0	2,630 ± 1,110	>4,000	>4,000	1,110 ± 410	>3.6
Ribavirin	µM	28 ± 0 <sup>c</sup>	ND <sup>d</sup>	ND	>4,100 <sup>e</sup>	>4,100	690 ± 97	>6.0
Infergen	µg/ml	0.012 ± 0.01	0.01 ± 0	0.019 ± 0.002	>10	>1.0	0.00057	>1,750

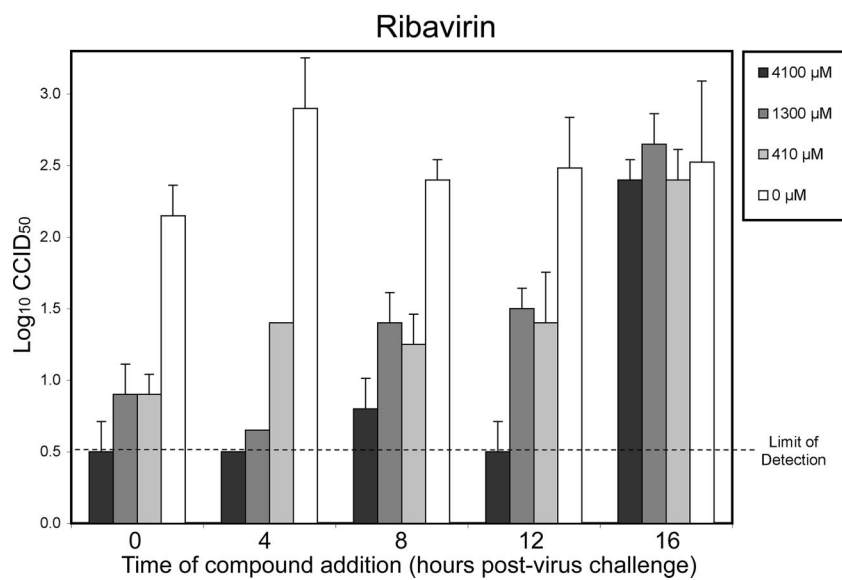
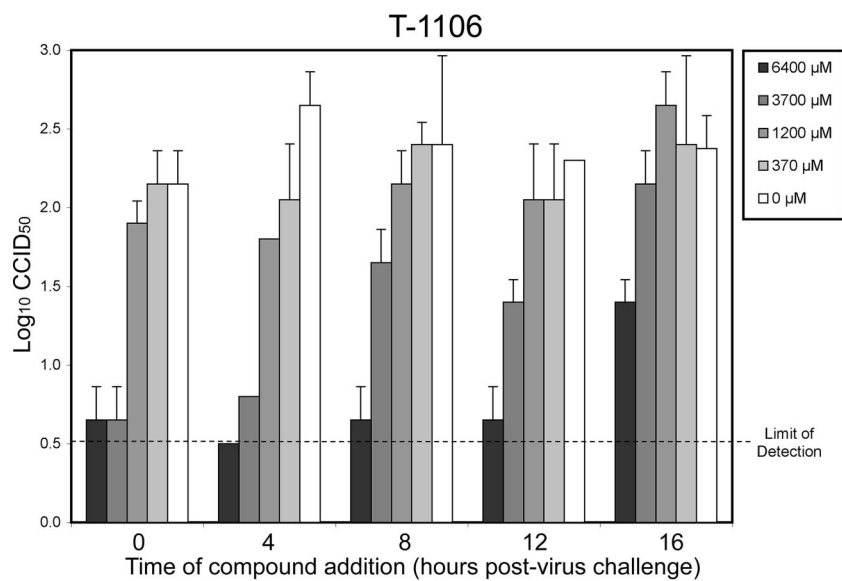
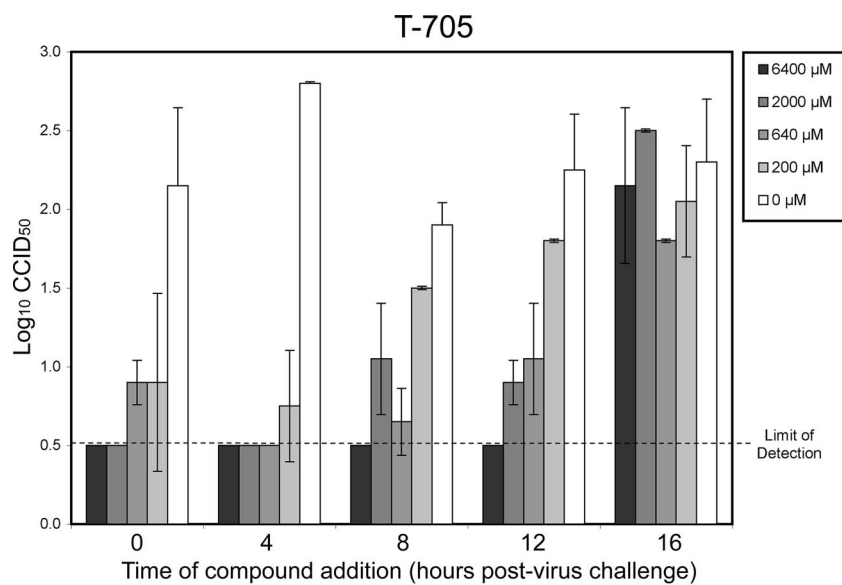
<sup>a</sup> Vero monolayers infected with  $10^{2.6}$  CCID<sub>50</sub>s of YFV strain 17D. Each assay was conducted at least twice, except where indicated, and the results are expressed as the means ± standard deviations.

<sup>b</sup> VYR, virus yield reduction assay.

<sup>c</sup> The assay was conducted once.

<sup>d</sup> ND, not done.

<sup>e</sup> As determined by visual inspection.



**Infectious cell culture assay.** Vero cells were cultured in 96-well flat-bottomed microplates 1 day before use. Tissue samples, obtained at necropsy from five infected hamsters from each group at 4 dpi, were homogenized and were then serially diluted at log concentrations ( $10^{-1}$  to  $10^{-8}$ ) in sterile culture medium. Dilutions of the tissue homogenate were added to microplates containing semi-confluent Vero cells. The plates were placed in a CO<sub>2</sub> incubator for 6 days, after which the cells were observed microscopically for a virus CPE. The virus titers in the samples were calculated on the basis of the tissue weight and the titer observed in dilutions of tissue samples.

**Statistical analysis.** Survival data were analyzed by Wilcoxon log-rank survival analysis (JMP software; The Statistical Discovery Software, SAS Institute, Inc.). All other statistical analyses were done by using a one-way Student *t* test.

## RESULTS

**Cell culture studies.** The EC<sub>50</sub>s of T-705 and T-1106 were evaluated against the 17D strain of YFV by evaluation of Vero cells for inhibition of the CPE. As reported previously, T-1106 was not active at concentrations below 370  $\mu$ M (10). Twofold dilutions of T-705 and T-1106, beginning at 4,000  $\mu$ M, were added to cells just prior to virus addition. T-705 had an average EC<sub>50</sub> of 180  $\mu$ M by visual inspection of the CPE and 270  $\mu$ M as determined by the NR dye uptake assay (Table 1). The EC<sub>50</sub>s of T-1106 were higher, with average concentrations of 1,080 and 1,800  $\mu$ M, as determined by visual inspection of the CPE and the NR dye uptake assay, respectively (Table 1). Ribavirin treatment in cell culture resulted in an EC<sub>50</sub> of 28  $\mu$ M, as determined by visual inspection. The EC<sub>50</sub> of ribavirin was lower than that of T-705 or T-1106 (Table 1).

The EC<sub>50</sub>s from initial cell culture experiments were confirmed by a luciferase-based assay that quantified the ATP levels in the cells as an indirect measure of cell viability. The average EC<sub>50</sub>s from two separate experiments with T-705 and T-1106 were 270 and 2,630  $\mu$ M, respectively (Table 1). The CC<sub>50</sub>s were >6,370 and >4,000  $\mu$ M for T-705 and T-1106, respectively.

To verify the antiviral activity observed by the CPE inhibition assay, a virus yield reduction assay was performed to determine the EC<sub>90</sub>s in Vero cells. The average EC<sub>90</sub> (from four separate experiments) of T-705 was 330  $\mu$ M, and that of T-1106 (from three separate experiments) was 1,110  $\mu$ M (Table 1). These concentrations are similar to the EC<sub>50</sub>s seen previously (Julander, unpublished data). With CC<sub>50</sub>s of >6,370 and >4,000  $\mu$ M for T-705 and T-1106, respectively, the SIs of these compounds indicate a range of very slight activity for T-1106 to moderate activity for T-705, as determined by the virus yield reduction assay (Table 1). Ribavirin and infergen were also included as positive controls, and they had SIs of >6.0 and >1,750, respectively.

Time-of-addition studies were conducted to determine the time critical to the antiviral efficacy of T-705 and T-1106 against YFV in Vero cells. Ribavirin was also included in these studies as a positive control. The initial release of virus progeny was first detected 24 h after infection of Vero cells with YFV strain 17D (data not shown). The compounds were added at

various concentrations at 0, 4, 8, 12, and 16 h after virus attachment; and the virus titer was assayed by the virus yield reduction assay at 24 h. Addition of T-1106 or T-705 at the highest concentration resulted in the inhibition of virus replication for up to 12 h after virus addition, while lower doses of the compounds reduced the amount of virus when they were added earlier in the replication cycle but had diminished efficacies at later time points (Fig. 2). In this study, ribavirin showed a virus reduction profile similar to the profiles of T-705 and T-1106, although ribavirin appeared to have an effect at lower doses initiated later in infection.

**Hamster studies.** Experiments were conducted to determine a minimal effective dose of T-705 for the treatment of hamsters infected with YFV. Animals were treated twice daily with various concentrations of T-705 administered p.o. and beginning 4 h prior to virus challenge (time -4 h). An initial experiment was conducted to evaluate concentrations of 100 and 400 mg/kg/day, and the higher dose was shown to significantly improve survival, liver virus titers, serum ALT levels, and weight change (Table 2; Fig. 3). Treatment of hamsters with 100 mg/kg/day did not significantly improve any of the disease parameters (Table 2; Fig. 3). These results were confirmed in a second experiment, in which significant improvements in the disease parameters were observed after treatment with 400 mg/kg/day but not with 100 mg/kg/day (Table 2).

An intermediate dose of 200 mg/kg/day was included in the second experiment, which proved to be effective in significantly improving the rate of survival (Table 2). The peak virus titer in the liver was also significantly reduced at this dose, as determined by QRT-PCR analysis of RNA extracted from liver samples taken 5 dpi from animals treated with T-705 according to the aforementioned schedule (Fig. 3). The weight change was not significantly improved, although there was a trend toward improvement (Table 2). Serum ALT levels were significantly reduced by treatment with T-705 at 200 mg/kg/day compared with the levels in placebo-treated mice (Table 2), but the serum AST level was not statistically reduced, despite a trend toward improvement (data not shown).

Greater efficacy was achieved with T-705 at 400 mg/kg/day than with T-705 at the 200-mg/kg/day dose, as determined by a greater reduction in the serum ALT level and also a significant improvement in weight change. The survival rates, however, were similar in both treatment groups. No signs of toxicity, including mortality, weight change, or morbidity, were apparent in uninfected hamsters treated with 400 mg/kg/day (Table 2).

T-1106 was administered to hamsters in parallel as a positive control. A significant improvement in the rate of survival was observed with a dose of 10 mg/kg/day (Table 2), which was lower than the previously reported minimal effective dose of 32 mg/kg/day (10); but despite the highly significant improvement in survival, no significant improvement in weight change or

FIG. 2. Time-of-addition study, in which T-705, T-1106, or ribavirin was added 0, 4, 8, 12, or 16 h after virus attachment. The supernatant was assayed for the initial level of virus production at 24 h after virus attachment by a virus yield reduction assay, and the results were compared with those of experiments in which no compound was added. Various doses of compound were tested at each treatment initiation time point, and the resulting CCID<sub>50</sub>s are shown. Tests were run in duplicate, and mean values are shown. Two independent experiments were conducted, and representative data are shown.



TABLE 2. Effect of T-705 administered p.o. on disease parameters for hamsters infected with YFV<sup>a</sup>

Expt no.	Treatment	Dose (mg/kg/day)	Toxicity controls		Infected, treated animals <sup>c</sup>			
			No. of animals alive/total no.	Mean wt change <sup>b</sup> (g) $\pm$ SD	No. of animals alive/total no.	MDD <sup>d</sup> $\pm$ SD	Mean serum ALT level (IU/liter) $\pm$ SD	Mean wt change (g) $\pm$ SD
1	T-705	400	3/3	5.3 $\pm$ 0.6	9/10***	6.0 $\pm$ 0.0	97 $\pm$ 53**	5.2 $\pm$ 5.9**
	T-705	100			3/10	7.7 $\pm$ 1.0	157 $\pm$ 59	-2.9 $\pm$ 6.6
	Saline				1/10	7.0 $\pm$ 1.2	183 $\pm$ 69	-5.2 $\pm$ 5.0
	Healthy controls		3/3	7.7 $\pm$ 1.2				
2	T-705	400			8/8***	>21.0 $\pm$ 0.0	63 $\pm$ 5**	5.8 $\pm$ 1.8**
	T-705	200			10/10***	>21.0 $\pm$ 0.0	128 $\pm$ 43*	2.2 $\pm$ 2.7
	T-705	100			0/10	7.1 $\pm$ 1.0	211 $\pm$ 47	-6.3 $\pm$ 3.2
	T-1106	32			8/8***	>21.0 $\pm$ 0.0	85 $\pm$ 17*	6.4 $\pm$ 3.1**
	T-1106	10			8/10***	8.0 $\pm$ 0.0	154 $\pm$ 41	0.2 $\pm$ 5.4
	Ribavirin	50			10/10***	>21.0 $\pm$ 0.0	103 $\pm$ 45*	2.4 $\pm$ 3.7
	Saline		2/2	8.5 $\pm$ 3.5	2/15	7.0 $\pm$ 1.4	173 $\pm$ 71	-3.1 $\pm$ 6.7
	Healthy controls		3/3	8.0 $\pm$ 2.0				

<sup>a</sup> T-705 was administered p.o. or T-1106 was administered i.p. twice daily for 8 days. Female hamsters (weight, 100 to 110 g) were challenged i.p. with 10<sup>2</sup> CCID<sub>50</sub> of hamster-adapted YFV strain Jimenez.

<sup>b</sup> Difference between weight on day 3 and that on day 6 after virus injection.

<sup>c</sup> \*\*\*,  $P < 0.001$  compared with the results for hamsters treated with placebo; \*\*,  $P < 0.01$  compared with the results for hamsters treated with placebo; \*,  $P < 0.05$  compared with the results for hamsters treated with placebo.

<sup>d</sup> MDD, mean days to death of mice dying prior to 21 dpi.

serum aminotransferase levels was observed when T-1106 was used at 10 mg/kg/day. Liver virus titers were significantly reduced after treatment with T-1106 at a dose of 32 mg/kg/day (Fig. 3).

Hamsters were treated with T-705 at a dose of 400 mg/kg/day beginning at -4 h or 1, 2, 3, 4, or 5 dpi to determine the effect of delayed treatment initiation on the outcome of disease. A slightly significant improvement in the rate of survival was observed when treatment was initiated as late as 3 dpi, although 20% of the hamsters died (Table 3). Complete survival was seen when treatment was initiated at 1 or 2 dpi, as well as at -4 h. Survival rates of 60 and 50% were observed

when treatment was initiated at 4 and 5 dpi, respectively, which was an improvement, although nonsignificant, compared with the 30% rate of survival for the placebo-treated animals. The mean no. of days to death of hamsters in the groups in which T-705 treatment was initiated at 3, 4, and 5 dpi was similar to that of the placebo-treated animals (Table 3).

Other disease parameters, including weight change and serum ALT and AST levels, were significantly improved in treated animals compared with those in placebo-treated animals when treatment began at -4 h and 1 and 2 dpi (Table 3). Some improvement was also observed with the later initiation of treatment, although this improvement was not significant, except for a slightly significant decrease in the serum ALT levels in hamsters treated beginning at 4 dpi (Table 3).

An interesting observation was made when the disease parameters for the surviving and nonsurviving hamsters from the groups in which treatment was initiated at 4 and 5 dpi were compared with those for the placebo-treated control animals. The surviving hamsters in the group in which treatment was initiated at 4 dpi had an average ALT level of 77 IU/liter, which was significantly lower than the serum ALT levels in the surviving hamsters in the placebo control group and which approached the background quantities (Table 4). A similar trend was seen with serum AST values; however, the average level was not significantly different from that for the placebo-treated animals. Survivors in the group in which treatment was initiated at 5 dpi had intermediate average serum ALT and AST levels of 132 and 153 IU/liter, respectively (Table 4), which were less than but not significantly different from the levels for the placebo-treated animals. The trend in weight change was similar to that seen with serum aminotransferase levels, with a 2.2-g weight gain in survivors from the group in which treatment was initiated at 4 dpi, a 2.8-g weight loss in survivors from the group in which treatment was initiated at 5 dpi, and a 10-g weight loss in hamsters treated with placebo

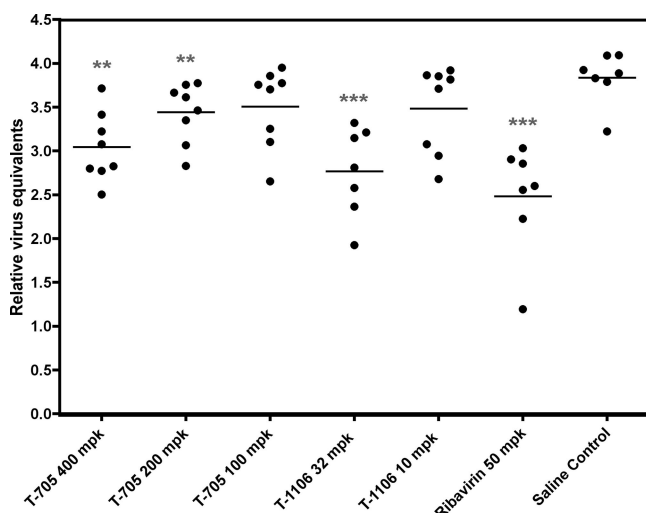


FIG. 3. YFV RNA levels detected by QRT-PCR on 6 dpi from liver tissue of hamsters treated with various concentrations of T-705, T-1106, or ribavirin. mpk, mg/kg/day; \*\*\*,  $P < 0.001$  compared with the results for the placebo group; \*\*,  $P < 0.01$  compared with the results for the placebo group.

TABLE 3. Effect of treatment with T-705 after virus exposure on disease parameters in hamsters challenged with YFV<sup>a</sup>

Treatment	Time of treatment initiation	Toxicity controls		Infected, treated animals <sup>c</sup>				
		No. of animals alive/total no.	Mean wt change <sup>b</sup> (g) $\pm$ SD	No. of animals alive/total no.	MDD <sup>d</sup> $\pm$ SD	Mean serum enzyme level (IU/liter) $\pm$ SD		Mean wt change (g) $\pm$ SD
						ALT	AST	
T-705	-4 h	3/3	1.0 $\pm$ 1.7	10/10***	>21.0 $\pm$ 0.0	75 $\pm$ 23**	52 $\pm$ 16**	2.8 $\pm$ 4.6**
T-705	1 dpi			10/10***	>21.0 $\pm$ 0.0	80 $\pm$ 32**	52 $\pm$ 28**	-0.3 $\pm$ 3.7**
T-705	2 dpi			10/10***	>21.0 $\pm$ 0.0	84 $\pm$ 47**	75 $\pm$ 85*	0.0 $\pm$ 7.8*
T-705	3 dpi			8/10*	7.0 $\pm$ 1.4	139 $\pm$ 71	137 $\pm$ 105	-4.1 $\pm$ 6.2
T-705	4 dpi			6/10	6.5 $\pm$ 0.6	115 $\pm$ 82*	131 $\pm$ 133	-3.0 $\pm$ 10.1
T-705	5 dpi			5/10	6.8 $\pm$ 0.8	157 $\pm$ 74	212 $\pm$ 118	-4.5 $\pm$ 6.1
Ribavirin	-4 h			10/10***	>21.0 $\pm$ 0.0	110 $\pm$ 61*	103 $\pm$ 99	-4.2 $\pm$ 5.7*
Saline	-4 h	3/3	0.7 $\pm$ 2.1	3/10	7.4 $\pm$ 1.8	188 $\pm$ 94	271 $\pm$ 169	-9.6 $\pm$ 6.4
Healthy controls		3/3	3.0 $\pm$ 0.0					

<sup>a</sup> T-705 was administered p.o. twice daily for 8 days at a dose of 400 mg/kg/day. Female hamsters (weight, 100 to 110 g) were challenged i.p. with 10<sup>2</sup> CCID<sub>50</sub>s of hamster-adapted YFV strain Jimenez.

<sup>b</sup> Difference between weight on day 3 and that on day 6 after virus injection.

<sup>c</sup> \*,  $P < 0.05$  compared with the results for the saline-treated controls; \*\*,  $P < 0.01$  compared with the results for the saline-treated controls; \*\*\*,  $P < 0.001$  compared with the results for the saline-treated controls.

<sup>d</sup> MDD, mean day to death of hamsters dying prior to 21 dpi.

(Table 4). This phenomenon was reversed in nonsurviving hamsters, in which animals treated with T-705 beginning at 4 dpi had a trend toward higher serum ALT and AST levels and greater weight loss than the animals treated with T-705 beginning at 5 dpi and the placebo-treated animals (Table 4).

## DISCUSSION

The anti-influenza virus agent T-705 was moderately active against YFV strain 17D in cell culture, with a mean EC<sub>90</sub> of 330  $\pm$  90  $\mu$ M and a CC<sub>50</sub> of >6,370  $\mu$ M. Compared with the SI of T-1106 in cell culture, the SI of T-705 was about 5.3-fold greater, indicating that T-705 has more potent anti-YFV activity in Vero cells. This dose, however, was almost 100-fold higher than the EC<sub>90</sub> of T-705 for the treatment of influenza virus infection in MDCK cells (21). T-1106 is poorly phosphorylated in various cell lines (7), which may explain the lower level of activity of T-1106 compared with that of T-705. A similar trend in the activities of T-705 and T-1106 was observed against the Jimenez strain of YFV in cell culture (data not shown), suggesting that the activities of T-705 and T-1106 against both virus strains used in this study are similar. Despite the high EC<sub>90</sub> of T-1106 in cell culture, this compound is highly

effective in the YFV hamster model, indicating the superior activation of this compound in the animal to that in Vero cells (10). The minimal effective dose for the treatment of influenza virus in a mouse model was sixfold lower than that in the YFV hamster model, indicating that the disparity in the EC<sub>90</sub>s seen in cell culture is not apparent in vivo and may simply be due to different rates of conversion (21).

Time-of-addition experiments demonstrated that the anti-YFV activity of T-705 occurs even at later stages of virus replication, which is similar to the results obtained with T-1106. These results are in agreement with previously published data involving time-of-addition studies with T-705 for the treatment of influenza virus infection (6), which supports the hypothesis that these compounds inhibit the polymerase of YFV. The efficacy of T-705 when it was administered beginning at 3 dpi also suggests that this compound acts at some time after virus attachment, entry into cells, and initial rounds of replication, further supporting the role of T-705 as an inhibitor of YFV polymerase. These and previously published results (19) suggest a possible similarity in the modes of action of T-1106 and T-705 with the mode of action of ribavirin for the treatment of YFV.

Hamsters treated with T-705 p.o. at doses of 200 and 400

TABLE 4. Comparison of surviving and nonsurviving hamsters infected with YFV and treated with T-705 initiated at 4 or 5 dpi and hamsters treated with placebo<sup>a</sup>

Treatment <sup>b</sup> and dpi of treatment initiation	Survivors				Nonsurvivors			
	No. of animals	Mean serum enzyme level (IU/liter) $\pm$ SD		Mean wt change (g) $\pm$ SD	No. of animals	Mean serum enzyme level (IU/liter) $\pm$ SD		Mean wt change (g) $\pm$ SD
		ALT	AST			ALT	AST	
T-705								
4 dpi	6	77 $\pm$ 41 <sup>c</sup>	64 $\pm$ 56	2.2 $\pm$ 3.7 <sup>c</sup>	3	231 $\pm$ 51	332 $\pm$ 23	-18.5 $\pm$ 3.5
5 dpi	5	132 $\pm$ 94	153 $\pm$ 134	-2.8 $\pm$ 7.3	4	189 $\pm$ 24	285 $\pm$ 15	-7.3 $\pm$ 2.5
Saline, -4h	3	177 $\pm$ 103	246 $\pm$ 195	-10.0 $\pm$ 4.6	4	196 $\pm$ 103	290 $\pm$ 175	-9.3 $\pm$ 8.2

<sup>a</sup> Serum ALT and AST levels and weight change were used as parameters for the comparison.

<sup>b</sup> Treatment with T-705 was at 400 mg/kg/day.

<sup>c</sup>  $P < 0.05$  compared with the results for the placebo-treated group.

mg/kg/day were significantly protected from death, whereas the placebo-treated controls had an 87% rate of mortality. The serum ALT level was also significantly improved in animals treated with T-705 at these two doses. Treatment with the higher dose of 400 mg/kg/day also resulted in a significant improvement in weight change, although a significant improvement was not seen with the 200-mg/kg/day dose. The dose of 200 mg/kg/day (1,270  $\mu$ mol/kg/day) used was approximately 11-fold higher than the highly effective dose of T-1106 that was used previously (32 mg/kg/day, or 120  $\mu$ mol/kg/day) (10), demonstrating the superior activity of T-1106 in the treatment of YFV in hamsters. This may be partially due to the different routes of administration, i.e., the i.p. route for T-1106 versus the p.o. route for T-705, although previous studies have shown that that activity of T-1106 given p.o. was similar to that of the drug given i.p. (10). Rates of conversion of T-1106 to the triphosphate form higher than those of T-705 have been observed in mammalian livers (7), which may account for the efficacy of T-1106 in the hamster model. It is possible that there are differences in the conversion and biodistribution of T-705 and those of T-1106.

An interesting phenomenon was observed in surviving and nonsurviving hamsters treated with T-705 beginning at 4 dpi compared with those treated with placebo. The surviving hamsters in the treated group had significantly lower serum ALT levels than the surviving hamsters in the placebo group, and the weight change in the surviving hamsters in the treated group was significantly different from that in the surviving hamsters in the placebo group. A reverse trend was observed for nonsurvivors, with higher average serum ALT and AST levels and greater weight loss for treated hamsters than for placebo-treated hamsters, although these data were not significantly different. It appears that T-705 treatment protects animals from death, unless there is overwhelming disease, which may be due to individual differences in immune response, viral fitness in the individual animal, or another unknown mechanism which allows the virus to overcome treatment. This finding also reinforces the importance of early treatment initiation, which is often a difficult prospect in human disease cases. A significant increase in overall survival was observed when hamsters were treated beginning at 3 dpi, indicating that this compound may be therapeutically useful.

T-705 is currently undergoing clinical trials for the treatment of influenza virus infection in humans (4). T-705 has also been shown to have broad-spectrum activity against several RNA viruses, including West Nile virus (18), Punta Toro and Pichinde viruses (8), and to a lesser extent, some alphaviruses (data not shown). If clinical trials with influenza virus are successful and T-705 is licensed for use for human therapy, it might be developed for the treatment of other viral infections as a second indication for treatment. Clinical trials with T-705 for the treatment of YFV or other acute RNA viral infections may not be feasible, but they would be of benefit if the compound was found to improve the outcomes of these viral infections in humans.

#### ACKNOWLEDGMENTS

We thank Robert Tesh for providing hamster-adapted YFV strain Jimenez. We also thank Luci Wandersee and Jeremy Strange for their work in the animal and cell culture laboratories.

This work was supported by contract NO1-AI-15435 and contract NO1-AI-30048 from the Virology Branch, NIAID, NIH.

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